Neutralization of HIV-1 by redirection of natural antibodies

Maria F. Perdomo*, Michael Levi†, Matti Sällberg*, and Anders Vahlne*‡

*Division of Clinical Microbiology, Karolinska Institutet, Karolinska University Hospital at Huddinge F-68, SE-141 86 Stockholm, Sweden; and †Tripep AB, Hälsovägen 7, SE-141 57 Huddinge, Sweden

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The great variability and high glycosylation of gp120 poses a great challenge for the design of a functional immune therapy. The binding region of the CD4 receptor to gp120, however, is well conserved and may constitute a target to limit viral entry and infectivity. Our strategy consists in using a preexisting pool of natural antibodies directed toward the gal(α1,3)gal disaccharide and to redirect it to HIV. We here show that using CD4-derived, gp120-binding, synthetic peptides chemically linked to gal(α1,3)gal can redirect these natural antibodies and improve the HIV-1 neutralizing activity of the CD4-derived peptides *in vitro*. Importantly, the binding of the CD4-gal(α1,3)gal peptides to HIV-1–infected cells conferred antibody-dependent cellular cytotoxicity after the addition of human sera. Thus, the temporary redirection of naturally occurring antibodies and their biological activities to a new antigen represents a completely new way of targeting a human disease.

ADCC | peptides | gal(α1,3)gal | CD4

Natural antibodies play an important role in the first line of defense against many viral and bacterial infections, providing a link between the innate and adaptive immune response (1). They have been shown to be important in the antibody-mediated opsonization of particles by macrophages and in the induction of the classic cascade of the complement (2). Moreover, these antibodies can trigger the activation of natural killer (NK) cells via CD16 recognition of the Fc fraction of the antibody and induce an antibody-dependent cellular cytotoxicity (ADCC) (3). In human serum, ≈1–8% of the total IgM and 1 to 2.4% of the total IgG recognize the epitope gal(α1,3)gal (4), which is part of a pentasaccharide present mainly on endothelial cells of all mammals except humans and old world monkeys (4–6). Thus, it would be very useful to temporarily be able to redirect this high-level preexisting antibody pool to a new antigen, which rapidly would increase the pool of biologically active antibodies with a predetermined specificity as an alternative to administering a monoclonal antibody.

To explore this possibility, we chose to target the receptor-binding region of the envelope protein (gp120) of HIV. The interaction between gp120 and its receptor, the CD4 molecule, is highly conserved and involves only a limited amount of residues. The envelope glycoprotein binds to 22 residues on the D1 region of the CD4 receptor located between amino acids 25 and 64, and the atomic interactions are well characterized. Most of the contact is established by amino acids of the CDR2-like region, and the most important residues are Phe 43 and Arg 59 (7–10).

A theoretical advantage of using molecules that mimic the CD4 receptor is that they should not induce the selection of mutants, since any changes in the binding region would be deleterious for the infectivity of the virus.

We here show that the chemical linking of gal(α1,3)gal to gp120-binding CD4-derived peptides generated adaptor molecules that redirected preexisting natural antibodies toward gp120. These redirected natural antibodies facilitated neutralization of HIV *in vitro* and mediated killing of HIV-infected lymphocytes by ADCC. Thus, the biological activity contained by the natural antibodies can be redirected to a new target using glycosylated synthetic peptides.

Results

Gal(α1,3)gal-Linked CD4-Derived Peptides Redirect Natural Antibodies to HIV-1. The gal(α1,3)gal disaccharide chemically linked to the side chain of an aspartic acid was coupled to each of six 15-aa long overlapping peptides corresponding to amino acids 25–64 of the D1 region of the CD4 receptor. The redirection of natural anti-gal(α1,3)gal antibodies to HIV-1 gp120 was first tested by ELISA. Binding of anti-gal(α1,3)gal antibodies to solid-phase bound gp120 was detected for all peptides down to a concentration of 10 ng/ml (≈5 nM) using a 1:10 or 1:20 dilution of human serum (Fig. 1). Further dilutions of the human serum did not display significant binding compared with negative controls. All peptides bound with similar effectivity to gp120 (Fig. 1). Human serum depleted of the anti-gal(α1,3)gal antibodies did not detect bound glycopeptide, confirming that antibodies bound to the plates were directed against the gal(α1,3)gal disaccharide.

Binding of one of the glycopeptides to surface-expressed gp120 was also shown by immunofluorescence using chronically infected ACH2 cells (Fig. 2). These cells express the gp120 molecule on their surface after stimulation with phorbol 12-myristate 13-acetate (PMA). Binding of the glycopeptide to the cells was visualized using Alexa Fluor-conjugated isolectin B4 (Molecular Probes), which specifically binds to the gal(α1,3)gal antigen (Fig. 2*A*) and also by using human serum from HIV-seronegative individuals and Alexa Fluor-conjugated goat antihuman IgG and IgM (Fig. 2*C*). In the absence of glycopeptide or anti-gal(α1,3)gal antibodies, no binding to ACH2 cells was seen (Fig. 2 *B* and *D*, respectively).

Natural Antibodies Can Neutralize HIV-1 Through Gal(α1,3)gal-Linked CD4 Peptides. Neutralization of the HIV IIIB/lymphadenopathy-associated virus (LAV) virus by the gal(α1,3)gal-linked CD4 peptides alone or by the peptides plus human serum was tested by three different neutralization assays.

First, a single round infectivity neutralization assay was performed using TZM-bl cells that contain a tat-responsive luciferase reporter gene with the presence of the HIV protease inhibitor indinavir in the cell culture medium. As a result, luminescence values in cell lysate after 48 h of incubation reflect infectivity of the virus added. The glycopeptides were tested alone (Fig. 3*A*) or in the presence of 10% inactivated human serum from healthy individuals (Fig. 3*B*). The glycopeptides alone inhibited virus infectivity between 40% and 50%. The addition of human serum increased the neutralization by 10–20% units, giving neutralization values for all the peptides >50% even at a concentration as low as 1 ng/ml (Fig. 3*B*). The levels of neutralization seen with the gal(α1,3)gal-linked CD4 peptides were within the same range as those seen with various neutralizing monoclonal antibodies (Fig. 3*C*).

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Conflict of interest statement: A.V. and M.S. own shares in Tripep AB, the company that holds the intellectual property. M.L. is an employee of Tripep AB

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‡To whom correspondence should be addressed. E-mail:anders.vahlne@ki.se.

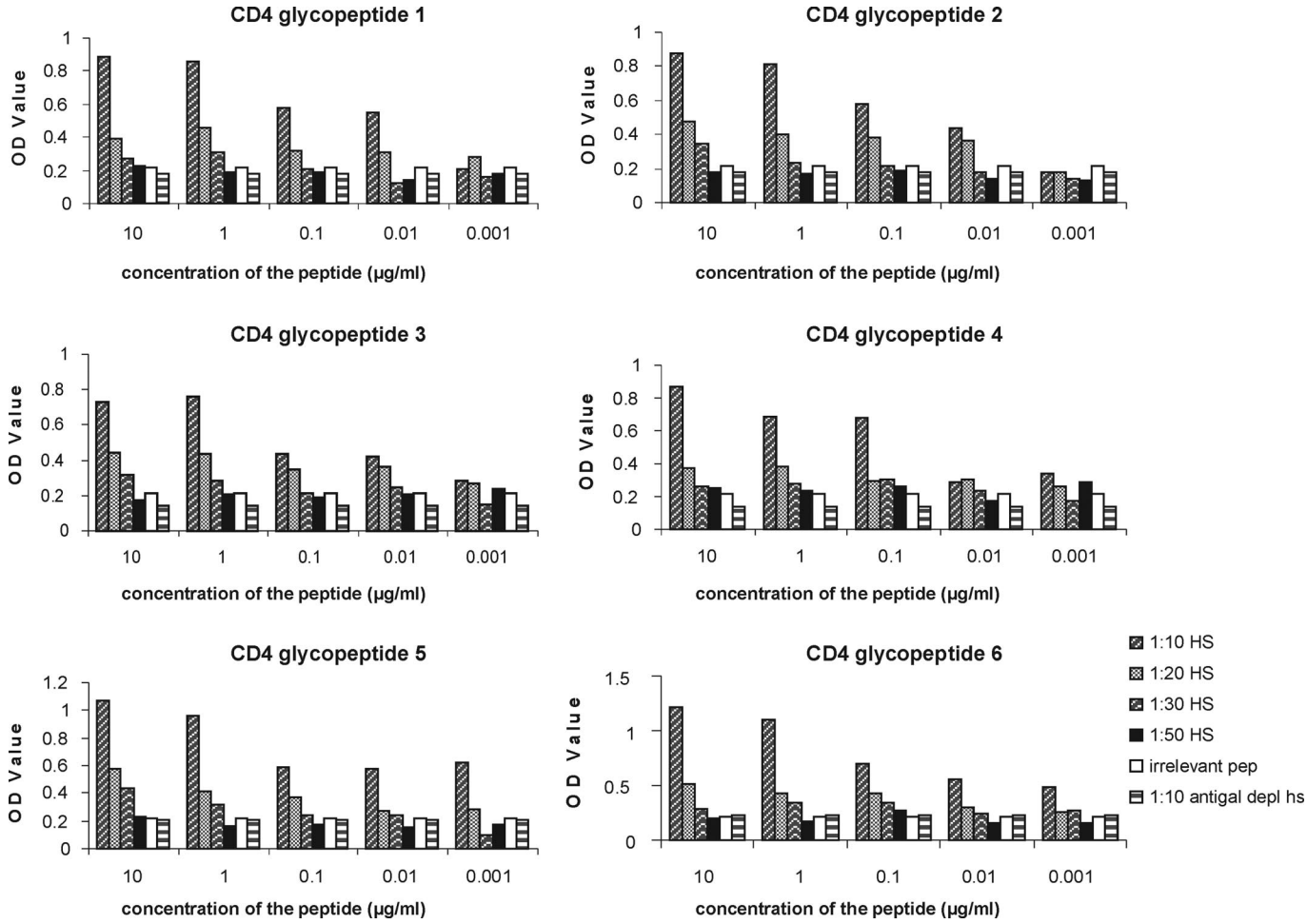

Fig. 1. Binding of CD4 glycopeptides. Each graph displays the binding of individual peptides on an ELISA assay in which binding of the glycopeptide-antibody complexes was assessed by using gp120-coated plates and several dilutions of human serum from healthy individuals. An irrelevant glycosylated peptide and gal(α1,3)gal-depleted serum were included as controls.

Second, we tested for neutralization based on the prevention of syncytia formation by HIV-1 infection of adherent U87 glioma cells expressing the CD4 receptor and the CXCR4 coreceptor (11). The fusion of infected cells is mediated by the gp120/gp41 proteins of HIV-1 expressed on the surface of infected cells that bind to CD4 and CXCR4 on neighboring cells. The addition of nonglycosylated or gal(α1,3)gal-linked CD4 peptides resulted in <40% neutralization, suggesting that the peptides themselves are inefficient in preventing syncytia formation (Fig. 3 *D* and *E*). When heat-inactivated human serum was added, the level of inhibition increased 5–10% units, suggesting that the presence of natural antibodies and gal(α1,3)gal-linked CD4 peptides alone has a limited effect on the inhibition of syncytia formation (Fig. 3 *F* and *G*). However, when noninactivated human serum was added together with the gal(α1,3)gal-linked CD4 peptides, the levels of inhibition of syncytia formation increased for all the glycosylated-CD4 peptides but not for the gal(α1,3)gal-linked control peptide (Fig. 3). Overall, the efficiency of the neutralizing activity was improved by 50–100% for all peptides when both the anti-gal(α1,3)gal antibodies and complement were present, strongly suggesting that the biological activity of the natural antibody had been redirected to HIV-1. Highest neutralization values were obtained with peptides 3, 4, and 5 (Fig. 3 *H* and *I*).

Finally, in the third neutralization assay, multiple rounds of replication, including cell-free transmission, were allowed. Reverse-transcriptase (RT) concentration was measured in cell supernatants at 11 days postinfection. All peptides neutralized the infection in a concentration-dependent manner; however, again, peptides 3, 4, and 5 displayed the highest values, giving a 90% neutralization at 1 μg/ml concentration and a >50% decrease in infectivity at a 0.01-μg/ml glycopeptide concentration (corresponding to ≈5 nM) at a 5 or 10% concentration of human serum (Fig. 3 *J* and *K*). In this assay, the presence of active complement gave an increase of neutralization by ≈10% as compared with heat-inactivated human serum (Fig. 3 *L* and *M*). Thus, in this assay, when multiple rounds of the whole viral life cycle were allowed, the effect of complement was less pronounced.

Gal(α1,3)gal-Linked CD4 Peptides Can Redirect Natural Antibodies to Kill HIV-Infected Cells by ADCC. The Fc region of antibodies attached to the surface of HIV-1–infected cells can be recognized by the CD16 receptor of NK cells and consecutively trigger a cytolytic response that will ultimately lead to the apoptosis of the targeted cell. To test whether anti-gal(α1,3)gal antibodies could induce ADCC of HIV-infected cells when bound to the glycopeptides, we performed flow cytometry on stimulated ACH2 cells expressing gp120. The target cells were preincubated with the glycopeptides, and the human serum, and freshly isolated NK cells were added at a final effector/target (E/T) ratio of 25:1. The ACH2 cells were detected by anti-CD5, and dying cells were detected by uptake of propidium iodide.

NK cell-mediated killing of HIV-1–infected cells was dependent

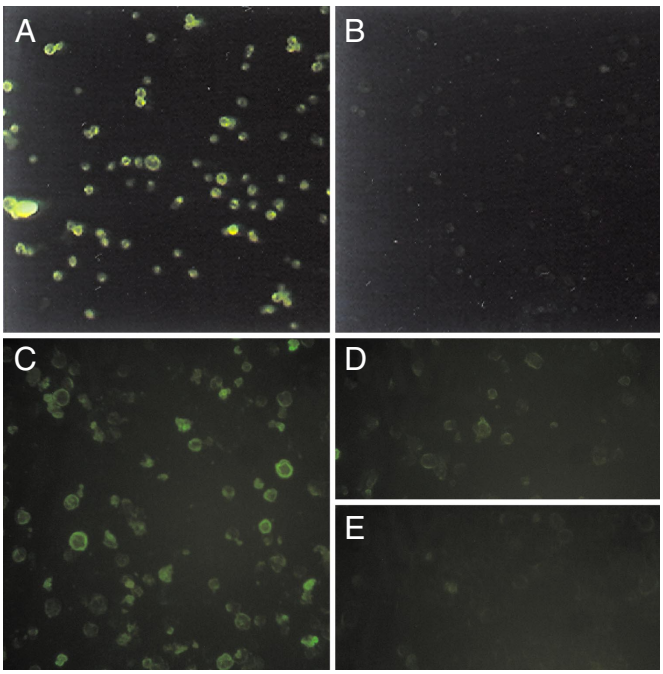

Fig. 2. Immunofluorescence. Binding of glycopeptide 4 to infected ACH2 cells was assessed using Alexa Fluor-conjugated isolectin B4 (*A*) as compared with samples in which no glycopeptide was added (*B*). The binding of anti-gal(α1,3)gal antibodies from an HIV-1–negative serum to glycopeptide 4 on the surface of ACH2 cells was detected with goat antihuman IgG and IgM Alexa Fluor-conjugated isolectin B4 (*C*) and, as a control, human serum depleted of the antigal antibodies (*D*) and unstimulated ACH2, which have a limited expression of gp120 in their surface (*E*).

on the concentration of the glycopeptides (data not shown), and lower concentrations ($<$10 μg/ml) did not display a significant portion of double-positive cells as compared with the background. Natural killer cell-dependent cytolysis was obtained with all gal(α1,3)gal-linked CD4 peptides but only in the presence of human serum (Fig. 4 *Upper*) strongly suggesting that the NK cell-binding activity of the natural antibodies had also been redirected to HIV-1–infected cells (Fig. 4). The results with glycopeptides 1 and 2 should be interpreted with caution, since these two glycopeptides exhibited some intrinsic toxicity when incubated with the ACH2 cells alone (Fig. 4 and data not shown). In conclusion, the gal(α1,3)gal-linked CD4 peptides were clearly able to redirect the ADCC activity mediated by the anti-gal(α1,3)gal antibodies to target HIV-1–infected lymphocytes.

Discussion

Long-lasting suppression of the HIV infection can today only be achieved by antiretroviral treatment. However, drug resistance, long-term side effects, and its high cost in a world in which the majority of infected individuals have no access to proper health care calls for the development of new strategies that could broaden the coverage and increase the compliance to therapy. Several approaches for HIV vaccine development have failed to provide a long-lasting immune response, mainly because the virus has developed several mechanisms of immune escape (12, 13). The great variability of the virus envelope glycoprotein gp120, its high level of glycosylation, and the fact that conserved epitopes are only exposed after binding of the receptor are some of the factors that limit the efficiency of an antibody response and pose a great challenge to the design of immune therapy (14, 15). The importance of an antibody response, however, has been underlined by the fact that passive immunization with neutralizing monoclonal antibodies has been shown to be able to suppress, at least temporarily, virus replication in infected individuals (16–18).

Virus-neutralizing antibodies are directed against viral surface structures, hindering the attachment of the virus to its receptor or coreceptor. The first step in the HIV replication cycle is the binding of its envelope glycoprotein to the CD4 molecule, which functions as the HIV receptor on susceptible lymphocytes. Upon binding, the gp120 molecule undergoes a series of conformational changes (19) that lead to the exposure of inner epitopes followed by binding to either the CCR5 or CXCR4 coreceptors and the initiation of the fusion process mediated by the gp41 molecule (20, 21). Attempts to block the early events of the virus replication cycle have targeted not only the receptor or coreceptor binding but the fusion of the virus envelope with the cell plasma membrane. To date, there are no attachment inhibitors and only one fusion inhibitor has been approved as a therapeutic agent (22).

It is well known that the attachment of HIV to its receptor can be blocked by soluble forms of the CD4 receptor (sCD4) (23–25). However, sCD4 molecules by themselves have not shown any efficacy in clinical trials, owing to a relative resistance of primary isolates to be neutralized by sCD4 (26) and possibly to the fact that the binding of sCD4 might facilitate direct binding of the virus to its coreceptors (27). However, it would certainly be beneficial to be able to use the selectivity of the CD4-gp120 interaction as an intervention strategy.

In the present report, we targeted the binding of HIV gp120 to its receptor by using synthetic peptides corresponding to the binding region of CD4. However, such peptides have no biological activity except for the mere blocking of the CD4-gp120 interaction. Here, we linked the CD4-derived gp120-binding peptides to the gal(α1,3)gal carbohydrate moiety, to which all humans have naturally occurring antibodies. This novel approach permitted us to redirect these preexisting naturally occurring anti-gal(α1,3)gal antibodies toward the gp120 molecule exposed at the surface of the virus and on the plasma membrane of HIV-infected cells, allowing for a completely new strategy for combating HIV-1 infection.

Of the six CD4 peptides, numbers 3, 4, and 5 were those giving the highest neutralization values, possibly because they contain the residues that have been described to make most of the contact with the gp120 molecule (7). CD4 peptides 3 and 4 contain amino acids 40–48, which make 63% of all interatomic contacts in gp120-CD4 binding, and they both contain the Phe-43 residue, which, alone, accounts for 23% of the interactions (7). Peptide 5 includes amino acids 45–59, and therefore contains another important residue, Arg-59, suggesting that this span of amino acids clearly is important for the attachment of the virus (28).

The desired immune event triggered by the gal(α1,3)gal-linked CD4 peptides is the binding of the natural anti-gal(α1,3)gal antibodies. It was envisioned that this binding could redirect the activation of the classic pathway of the complement cascade toward HIV-1. To evaluate this, we tested the gal(α1,3)gal-linked CD4 peptides in the presence of both untreated and heat-inactivated human serum. We found that the presence of complement contributed to a further decrease of 5–15% units in HIV infectivity. It is important to point out, however, that the cytolytic effect of complement might be masked by complement inhibitory proteins on the cell surface of the target cells and in the serum (29).

In addition to complement binding, we could show that the gal(α1,3)gal-linked CD4 peptide-mediated binding of anti-gal(α1,3)gal antibodies to HIV-infected cells could induce ADCC. NK cells have been implicated in both antibody-dependent (30) and -independent recognition (31, 32) of the gal(α1,3)gal epitope, although the latter remains controversial (33, 34). To address this question, we tested the glycopeptides in the presence or absence of human serum. A clear cytolytic effect was only seen after the addition of human serum. The binding of this complex to the surface of infected cells rendered the lymphocytes sensitive to NK cell-mediated killing by ADCC. All glycopeptides, except for num-

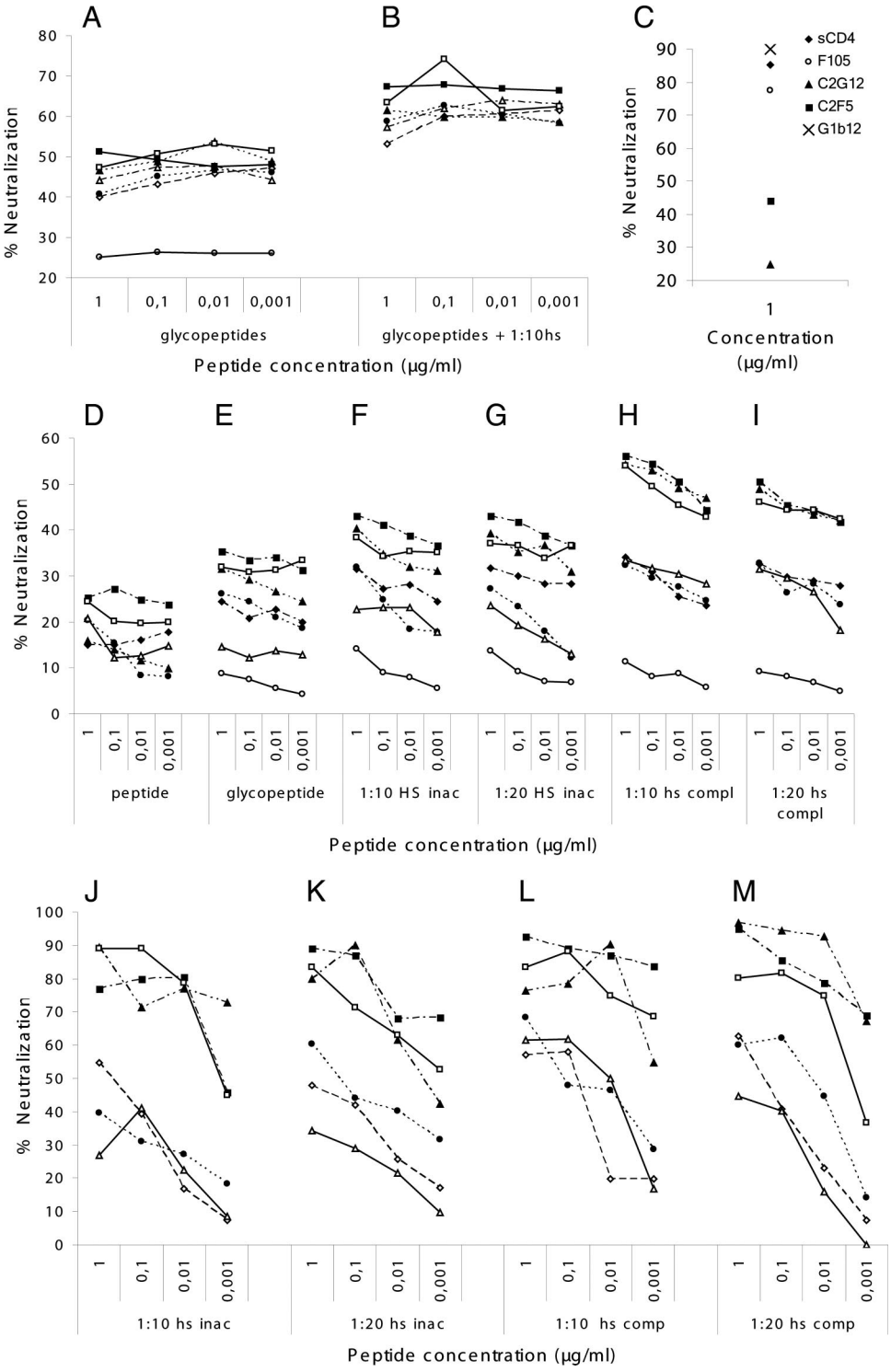

Fig. 3. Neutralization assays. Effect of the glycopeptides on viral entry and infectivity was tested on a single-round infectivity assay on TZM-bl cells (*Top*), a syncytia inhibition assay on U87 cells (*Middle*), and a multiple round infectivity assay on H9 cells (*Bottom*). In the first assay, the glycopeptides alone and a scramble CD4 peptide were tested as a control (*A*) but also in the presence of a 1:10 dilution of inactivated (inac) human serum (hs) (*B*). The standard deviation range was between 1% and 10% except for peptide 6 and scramble peptide; in these cases, it was ≈15%. (*C*) In the same setting a set of antibodies of known activity together with a soluble CD4 were run for comparison. In the second assay, the effect on the cytopathicity of the virus was studied with the nonglycosylated (*D*) and glycosylated (*E*) peptides, and the latter were tested in the presence of 1:10 (*F*) and 1:20 (*G*) dilutions of inac hs from healthy individuals. Blocks *H* and *I* correspond to the testing of the glycopeptides in the presence of a 1:10 or 1:20 dilution of non–heat-inactivated human serum (hs) to evaluate the role of the complement. Each peptide concentration was run in quadruplicate in the same plate and in five consecutive plates. Represented is the median value of the calculated ratio between treated and untreated infected cells obtained from 20 separate observations. In the third neutralization assay, the glycopeptides were tested in the presence of a 1:10 (*J*) or 1:20 (*K*) dilution of inactivated human serum and 1:10 (*L*) and 1:20 (*M*) dilutions of untreated human serum. The RT concentration was measured in cell supernatant 11 days postinfection. The standard deviation ranged between 5% and 15% for all peptides, being generally higher at lower concentrations of the glycopeptides. Represented is the percentage of neutralization, calculated as the ratio between treated samples and infected cells in the presence of hs. Peptide 1 (open diamonds), peptide 2 (filled circles), peptide 3 (filled triangles), peptide 4 (filled squares), peptide 5 (open squares), and peptide 6 (open triangles).

ber 6, mediated potent NK cell cytotoxicity on HIV chronically infected ACH2 cells by the redirection of the Fc-binding activity of the natural anti-gal(α1,3)gal antibodies to HIV-1.

The concentration of glycopeptides needed for ADCC was considerably higher than for HIV neutralization. This would possibly be attributable to low avidity of the glycopeptides and that neutralization is achieved by both the gal(α1,3)gal-linked CD4 peptides alone and when they are combined with human serum.

Taking advantage of the innate immune response could be of particular significance in pathological findings in which the cellular and humoral immune responses are compromised, such as in HIV, and it is of importance that two biological activities (i.e., both the complement binding and Fc binding) of the natural antibodies could be redirected by the gal(α1,3)gal-linked CD4 peptides. However, other processes of this primary response, such as opsonization of the peptide/antibody complexes, together with the role of the phagocytic cells need to be explored.

To optimize the gal(α1,3)gal-linked CD4 peptides, modifications to the peptide sequence in addition to other oligosaccharides such as blood group antigens could be explored.

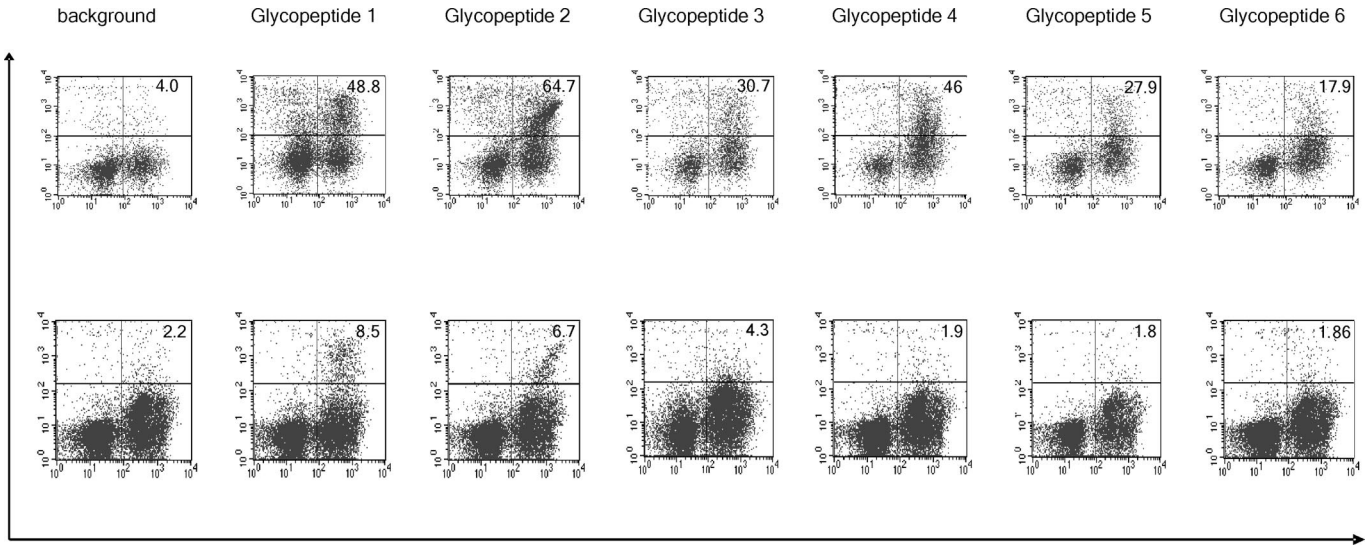

Fig. 4. Antibody-dependent cellular cytotoxicity. Chronically infected ACH2 cells were pretreated with 10 μg/ml glycopeptides (*Upper and Lower*) and a 10% concentration of inactivated human serum (*Upper*) before the addition of freshly isolated NK cells (E/T ratio = 25:1). The CD5 staining of target cells is represented in the *x* axis, whereas cell cytotoxicity was detected by staining with propidium iodide and is presented in the *y* axis. The figures listed in the *Upper Right* quadrant represent the percentage of ACH2 cells that stained positively for both dyes (i.e., the level of cytotoxicity).

In the present study, the effect of the CD4-derived glycopeptides was tested on the HIV IIIB/LAV virus strain only. This laboratory strain is well adapted to cultured lymphocytes and has been shown to be more susceptible to neutralization than primary isolates (35). For assessing the potential clinical use of gal(α1,3)gal-conjugated peptides, further experiments are needed, including analyzing their effect on other HIV laboratory strains and on primary isolates of HIV. Furthermore, since all animals except apes and old world monkeys express the gal(α1,3)gal epitope on their endothelial cells, *in vivo* studies on these glycopeptides can only be performed in gal(α1,3)gal knockout animals. However, the aim of the present study was to test whether it was possible to redirect natural antibodies with maintained biological activity to viruses or virus-infected cell surfaces using short gal(α1,3)gal-conjugated peptides. This was indeed the case, and such a strategy might therefore be used for other infectious diseases or other situations in which antibodies could be of potential benefit, such as in cancer therapy.

Methods

Peptide Synthesis. Six peptides derived from the binding region of the CD4 receptor to the gp120 molecule were synthesized using the peptide synthesizer Syro II (MultiSyntech) and standard Fmoc chemistry. The peptides were left still attached to their solid supports (resins), with side-chain protecting groups and free amino ends. Each peptide was 15 aa long, and they overlapped by 10 aa.

The disaccharide galα1–3galβ1-O $(CH_2)_3NH_2$ (Lectinity) was coupled to the side chain of an aspartic acid (NovaBiochem) with protected C- and N-terminal ends, creating a peptide bond between the sugar amino group and the amino acid side chain carboxyl group. A molar excess of amino acid to sugar between 2:1 and 4:1 was used, with a sugar concentration between 30 and 50 mM. The sugar/amino acid coupling took place for 3–5 h at room temperature or 37°C under constant shaking. The glyco-amino acid was then purified by reversed-phase HPLC (HPLC system LaChrom; Merck-Hitachi) using an acetonitrile gradient and the column Kromasil 100, C18, 250 $\times$ 10 mm (ChromTech). The purified glyco-amino acid was lyophilized in the freeze-dryer Heto Drywinner DW 1.0–60E (Heto).

To allow coupling to the CD4 peptides, the dried glyco-amino acid C terminus was deprotected by dissolving the glyco-amino acid in 2–7 ml of 95% TFA and incubating it for 1–3 h at room temperature under constant shaking. After that, the deprotected glyco-amino acid was purified by reversed-phase HPLC.

The deprotected glyco-amino acid was coupled to the N-terminal ends of the six resin-bound CD4 peptides, creating peptide bonds between the glyco-amino acid carboxyl group and the peptide amino groups. A molar excess of glyco-amino acid to peptide of 3:1 was used, with a glyco-amino acid concentration between 0.05 and 0.14 mM. The glyco-amino acid/peptide coupling took place for 4–6 h at 37°C under constant shaking. Consequently, the glycopeptide N-termini and side-chains were deprotected, and the glycopeptides were cleaved off their solid supports. After precipitation in ether and drying in air overnight, the glycopeptides were purified by reversed-phase HPLC and lyophilized.

The glycopeptides' molecular weights were verified by MS analysis (performed by Protein Analysis Centre at the Karolinska Institute).

ELISA Immunoassay. Binding of the peptides to the gp120 molecules and binding of the antibodies to the disaccharide were tested by enzyme immunoassay. Ninety-six–well microtiter plates (NUNC; Polysorp) were coated with LAV gp120 molecule (PerkinElmer) at 1 μg/ml in 50 mM carbonate-bicarbonate buffer and incubated for 1 h at 37°C. Plates were then blocked with a 2% solution of human serum albumin (Calbiochem) in PBS-A and incubated for 2 h at 37°C. After washing, the glycosylated peptides were added at different concentrations and incubated for 1 h at 37°C. Then, AB type human serum from healthy individuals (Cambrex) was added at different dilutions and incubated for 1 h. A polyvalent antihuman antibody HRP conjugate (Dako) was added and incubated for 1 h at 37°C. o-Phenylenediamine dihydrochloride (OPD) was used as a substrate, and plates were read at 490 nm after 30 min of incubation at room temperature in the dark.

Immunofluorescence. Binding of glycopeptide 4 to the gp120 molecule was also evaluated by immunofluorescence using chronically infected ACH2 cells that were stimulated with 50 nM PMA 48 h before the performance of the assay to ensure expression of the gp120 molecule on the cell surface. On the day of the assay, 20,000 cells were counted and allowed to dry on a microscope slide; they were then fixated with a 3% solution of paraformaldehyde at pH 7.0. The slide surface was blocked with a 2% solution of human serum albumin (Calbiochem) for 30 min in a humidified chamber at 37°C; thereafter, the glycopeptide was added at a concentration of 5 μg/ml and incubated for 1 h at 37°C in a humidified chamber. A 1-μg/ml solution of Isolectin B4 Alexa Fluor 488 conjugated (Molecular Probes, Invitrogen) was added to the cells to detect the bound peptide and incubated for 1 h at 37°C. In a second assay that aimed to visualize the glycopeptide-antibody complex, after the incubation of the peptide, a 10% dilution of human serum from HIV-seronegative individuals (Cambrex) was added and incubated for 1 h. Then, a 2-μg/ml concentration of antihuman IgG and IgM Alexa Fluor conjugated (Molecular Probes, Invitrogen) was added and incubated for 1 h. Cells were checked under a standard fluorescence microscope.

Neutralization Assay Using TZM-bl Cells. The TZM-bl cells were obtained through the National Institutes of Health (NIH) AIDS Research and Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), NIH: TZM-bl from J. C. Capes, X. Wu, and Tranzyme. Briefly, 10,000 cells per well were preseeded in a 96- well plate and incubated overnight at 37°C. The next day, the glycopeptide and 150 50% tissue culture infectious doses of the IIIB virus were preincubated for 1.5 h at 37°C; thereafter, the glycopeptide/HIV mixture was

added to the cells together with a 1:10 dilution of inactivated AB human serum obtained from healthy individuals (Cambrex). The cells were incubated with advanced DMEM containing 1 mM indinavir in a final volume of 200 μl per well for 48 h. Each tested concentration of glycopeptide was run in four replicates on the same plate. The luciferase activity in individual wells was measured using the Bright Glo Luciferase Kit (Promega) according to the manufacturer's protocol. The percentage of fusion inhibition was calculated as 1 minus the ratio of treated wells versus untreated-infected wells multiplied by 100.

Monoclonal antibodies G1b12, C2G12, F105, and F425 and soluble CD4 were tested under the same protocol, and thus served as positive controls (36, 37). These reagents were obtained through the NIH AIDS Research and Reagent Program, Division of AIDS, NIAID, NIH.

Syncytia Counting Neutralization Assay. The method was previously described by Shi *et al.* (11) in 2002 and is based on U87 cells that express the CD4 receptor and the CXCR4 coreceptors. Cells were seeded into 48-well plates (Costar, Corning) at a concentration of 10^5 cells per well 1 day before infection. On the day of infection, glycopeptides, pooled AB type human serum from healthy individuals (Cambrex), and previously titrated HIV IIIB virus were diluted in culture medium and preincubated in a separate 48-well plate for 1 h at 37°C. The peptides were analyzed at different concentrations to evaluate the minimum dose required for antiviral activity. Two hundred microliters of each dilution was distributed into triplicate wells containing the U87 $CD4^+$-$CXCR4^+$ cells and incubated for 12 h, after which medium was removed and 1 ml of DMEM medium was added to the cells. The positive controls were infected cells in the presence or absence of human serum, and negative controls consisted of uninfected cells in the presence or absence of human serum and an irrelevant glycosylated peptide. At day 5, the cells were fixated with methanol-acetone (1:1). The number of plaque-forming units was determined after hematoxylin staining, and the index was extracted from the ratio between infected cells treated with the peptides and human serum and cells infected with the virus in the presence of human serum. Each peptide was tested at four different concentrations, in quadruplicate in the same plate and in five different plates (i.e., each peptide was run in 20 wells at each concentration).

Infectivity Assay. The infectivity assay was performed on H9 cells that were infected with HIV IIIB virus. All peptides were run at four different dilutions, and each dilution was run in quadruplicate together with 10% or 5% of pooled AB human serum from healthy individuals. (Cambrex).

H9 cells were infected with 100 50% tissue culture infectious doses of the HIV IIIB virus and incubated for 1.5 h at 37°C, after which they were centrifuged, the supernatant was discarded, and the cells were resuspended in RPMI medium for a final concentration of 200,000 cells per ml.

One 48-well plate was run per glycopeptide, containing the four dilutions of the glycopeptide and the four replicates of each one of them, plus a positive control of infected cells (with and without human serum) and a negative control of noninfected H9 cells. Each well contained 500 μl of the infected cells plus 500 μl of the diluted peptide and the chosen dilution of the human serum.

Plates were incubated at 37°C plus 5% CO_2 until day 7, when 500 μl of supernatant was collected from each well and stored at $-20°$ until further analysis. An additional 500 μl of fresh RPMI medium with the appropriate dilution of the peptide plus the human serum was added to the wells and incubated at 37° until day 11, when the supernatant was measured for RT activity (Cavidi) according to the manufacturer's protocol.

ADCC. Cell cytotoxicity was evaluated by flow cytometry in a FACScalibur Flow Cytometer (BD Biosciences) using HIV chronically infected ACH2 cells and freshly isolated NK cells (3H Biomedical). The ACH2 cells were stimulated with 50 mM PMA (Sigma) 48 h before the assay to induce virus production. On the day of the assay, the ACH2 cells were preincubated for 2 h with the diluted peptide and 10% human serum AB from healthy individuals (Cambrex). Then, NK cells were added to a final E/T ratio of 25:1 in a final volume of 400 μl, and the cells were incubated for 4 h at 37°C. During the final 15 min of incubation, the ACH2 cells were stained by adding 5 μl per sample of anti–CD5-APC conjugated antibody (BD Biosciences), and as a marker of cell death, propidium iodide (Invitrogen) was added at 1 μg/ml. Samples were analyzed using the Cell Quest Program, and ACH2 cells were gated by size.

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